JOURNAL OF BACTERIOLOGY, Jan. 1979, p. 153-160
0021-9193/79/01-0153/08$02.00/0

Vol. 137, No. 1

Hydrogen-Dependent Nitrogenase Activity and ATP Formation in *Rhizobium japonicum* Bacteroids†

DAVID W. EMERICH,[1]* TOMÁS RUIZ-ARGÜESO,‡ TE MAY CHING,[2] AND HAROLD J. EVANS[1]

Laboratory for Nitrogen Fixation Research,[1] and Department of Agronomic Crop Science,[2] Oregon State University, Corvallis, Oregon 97331

Received for publication 25 October 1978

Rhizobium japonicum 122 DES bacteroids from soybean nodules possess an active H_2-oxidizing system that recycles all of the H_2 lost through nitrogenase-dependent H_2 evolution. The addition of 72 μM H_2 to suspensions of bacteroids increased O_2 uptake 300% and the rate of C_2H_2 reduction 300 to 500%. The optimal partial pressure of O_2 was increased, and the partial pressure of O_2 range for C_2H_2 reduction was extended by adding H_2. A supply of succinate to bacteroids resulted in effects similar to those obtained by adding H_2. Both H_2 and succinate provided respiratory protection for the N_2-fixing system in bacteroids. The oxidation of H_2 by bacteroids increased the steady-state pool of ATP by 20 to 40%. In the presence of 50 mM iodoacetate, which caused much greater inhibition of endogenous respiration than of H_2 oxidation, the addition of H_2 increased the steady-state pool of ATP in bacteroids by 500%. Inhibitor evidence and an absolute requirement for O_2 indicated that the H_2-stimulated ATP synthesis occurred through oxidative phosphorylation. In the presence of 50 mM iodoacetate, H_2-dependent ATP synthesis occurred at a rate sufficient to support nitrogenase activity. The addition of H_2 to H_2 uptake-negative strains of *R. japonicum* had no effect on ATP formation or C_2H_2 reduction. It is concluded that the H_2-oxidizing system in H_2 uptake-positive bacteroids benefits the N_2-fixing process by providing respiratory protection of the O_2-labile nitrogenase proteins and generating ATP to support maximal rates of C_2H_2 reduction by oxidation of the H_2 produced from the nitrogenase system.

ATP-dependent H_2 evolution by nitrogenase may consume up to 40% of the energy supplied to the N_2-fixing process in legumes (23). This represents a significant loss of energy and, thus, a potential limitation of the amount of fixed nitrogen available to the plant. An H_2-recycling system has been identified in certain legume-*Rhizobium* symbioses (4, 7, 11-13, 17, 18, 22), in blue-green algae (cyanobacteria) (5, 6, 19, 25), and in *Azotobacter chroococcum* (24, 27) that may regain some of the energy lost as H_2 from the N_2 fixation process. The presence of an H_2-oxidizing hydrogenase in N_2-fixing bacteria was reported by Phelps and Wilson (20) in 1941, and esterification of phosphate concomitant with the H_2 uptake in *Azotobacter* was observed by Hyndman et al. (14) in 1953. The possible significance of the H_2-recycling process was not fully realized before Dixon's (11-13) investigation of the hydrogenase system in bacteroids from *R. leguminosarum*. Dixon (13) postulated that the H_2 uptake system in bacteroids functioned as follows: (i) as a scavenger of O_2; (ii) as a mechanism which prevents H_2 inhibition of nitrogenase; and (iii) as a process whereby some of the energy lost via H_2 evolution from the nitrogenase system may be recovered through H_2 oxidation. Consumption of O_2 through the oxyhydrogen reaction in blue-green algae (6) and *Azotobacter* (27) protected the nitrogenase systems from O_2 damage. H_2-dependent ATP formation has been reported in blue-green algae (5, 19), *A. chroococcum* (27), and a particulate preparation of *R. leguminosarum* bacteroids (13). Also, H_2 utilization via the hydrogenase system in blue-green algae may provide reductant for nitrogenase (5). No evidence for H_2-dependent reductant supply to bacteroid nitrogenase has been obtained because bacteroids, unlike free-living organisms that can be experimentally deprived of carbon substrates, may contain carbon reserves up to 50% of their total dry weight (15).

In this paper we report that the H_2-oxidizing hydrogenase in *R. japonicum* strain USDA 122 DES bacteroids (referred to as *R. japonicum* 122 DES) provided a mechanism for respiratory

† Paper no. 4940 from the Oregon Agricultural Experiment Station, Corvallis, OR 97331.

‡ Present address: Department of Microbiology, Polytechnic University, Madrid, Spain.

protection of nitrogenase, increased the ATP supply, and supported nitrogenase activity.

MATERIALS AND METHODS

Soybean plants (*Glycine max* cv Wilkin) were grown, nodules were harvested, and bacteroids were prepared as described by Klucas et al. (15), with the exception that seeds were inoculated at planting with inoculum prepared from sterile peat and *R. japonicum* 122 DES as described by Vincent (26). All nodules produced by this procedure contained an active H_2 uptake system and therefore were not contaminated with strains of *R. japonicum* which lack the H_2 uptake complex. Strain USDA 122 DES is a small-colony derivative isolated from USDA 122 by the method of Kuykendall and Elkan (16). Plants were harvested after 30 days, and nodules were freed of perlite and rinsed in distilled water. Bacteroids were prepared by macerating 50 g of fresh nodules in an Omni-Mixer with 17 g of polyvinylpolypryrolidone and 250 ml of 0.05 M potassium phosphate buffer containing 0.2 M sodium ascorbate at pH 6.8. After maceration and passage through cheesecloth, the bacteroids were centrifuged at 9,000 × *g* for 15 min. The pelleted bacteroids were carefully separated from the polyvinylpolypryrolidone layer and resuspended in 50 mM potassium phosphate buffer containing 2.5 mM $MgCl_2$ at pH 7.0. The washing procedure was repeated three times, and bacteroids were finally resuspended in 50 ml of 50 mM *N*-2-hydroxyethyl piperazine-*N'*-2-ethanesulfonic acid (HEPES) buffer containing 2.5 mM $MgCl_2$ and 1 mM potassium phosphate at pH 7.5. Bacteroids were prepared anaerobically from fresh nodules, unless otherwise stated, and were used on the same day that they were prepared. After resuspension in HEPES-$MgCl_2$-K_2HPO_4 buffer, aerobically prepared bacteroids were flushed with argon and stored anaerobically. Bacteroids from an H_2 uptake-negative strain, *R. japonicum* USDA 117, were prepared from fresh nodules on Wilkin soybeans as described for USDA 122 DES. These bacteroids were used as controls. Acetylene reduction assays were performed in 20-ml bottles containing 2.45 ml of HEPES-Mg-phosphate buffer at pH 7.5. Bottles were sealed with rubber serum stoppers, evacuated, and refilled with argon three times and finally filled with H_2, O_2, and C_2H_2 as indicated and sufficient argon to obtain 1 atm. Except for Fig. 2, the appropriate volume was removed from the bottles containing 1.0 atm of argon and replaced with H_2, O_2, and when necessary C_2H_2 to obtain the desired partial pressure of each respective gas. The gas compositions for assays shown in Fig. 2 were obtained by adding, with the use of a syringe, the correct volume of each gas to an evacuated bottle. The bacteroid suspension (0.05 ml) was added to initiate the assays, and then vials were placed in a water bath at 23°C with shaking at 150 cycles per min.

A 0.5-ml gas sample was withdrawn from each reaction bottle for C_2H_2 reduction activity after 15 and 30 min of incubation. C_2H_4 formation was measured on a Hewlett-Packard HP-5830A gas chromatograph equipped with a column of Porapak R (1.8 m by 3.2-mm diameter) at 47°C. N_2 was used as a carrier gas at a flow rate of 40 ml/min. C_2H_4 formation was linear for at least 1 h.

H_2 and O_2 uptake rates were determined amperometrically as described by Hanus et al. (F. J. Hanus, K. R. Carter, and H. J. Evans, Methods Enzymol., in press). H_2 and O_2 were added to the electrode chamber (2.8-ml volume) as H_2- or O_2-saturated HEPES-Mg-phosphate buffer solutions. Initial concentrations of H_2 and O_2 in amperometric assays were typically 26 and 22 μM, respectively. Time courses of H_2 and O_2 uptake measured amperometrically were linear under the conditions in which the experiments were conducted (17).

Bacteroid samples for nucleotide determinations were prepared as described for C_2H_2 reduction assays. After incubation for periods indicated in the legends, the assays were terminated by rapid addition of 2.4 ml of ice-cold 0.8 M perchloric acid and immediately immersed in an ice bath at 0 to 2°C. Nucleotides were extracted from the bacteroid suspensions with vigorous shaking at 0°C for at least 30 min, and each sample was centrifuged at 15,000 × *g* for 10 min. The supernatant was frozen at −80°C until the determinations were made. For the assays, supernatants were thawed quickly and placed in an ice bath, and then 1.0 ml of 0.1 M HEPES buffer, pH 7.5, was added and the pH was adjusted to 6.5 to 7.0 with 3 N KOH. Neutralized samples were diluted 10-fold in ice-cold 25 mM HEPES-acetate buffer at pH 7.5. ATP, ADP, and AMP contents of diluted samples were measured by the luciferin-luciferase method as described by Ching and Ching (8). The hypotonic grinding and washing buffers destroyed contaminating plant mitochondria in the bacteroid suspensions. No mitochondrial band was observed in a sucrose density gradient (9).

H_2, O_2, and argon of the highest purities commercially available were obtained from Airco Industrial Gases, Vancouver, Wash. C_2H_2 was generated from CaC_2 and water. HEPES, iodoacetate, and carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) were purchased from Sigma Chemical Co. Luciferin and luciferase were prepared from Sigma FTE-50 firefly tails. All salts were analytical grade.

RESULTS

Effect of H_2 and succinate on C_2H_2 reduction and O_2 uptake. The rate of C_2H_2 reduction by bacteroids is dependent upon the partial pressure of O_2 (1, 3, 28). The effect of the partial pressure of O_2 on C_2H_2 reduction by *R. japonicum* 122 DES bacteroids is shown in Fig. 1. Consistent with other reports (2, 21), the addition of succinate stimulated the rate of C_2H_2 reduction. Succinate also enabled the organism to carry out C_2H_2 reduction at increased partial pressures of O_2 (Fig. 1). Additions of H_2 to the gas phase also stimulated C_2H_2 reduction activity and extended the range of O_2 partial pressures at which C_2H_2 reduction occurred. Saturating concentrations of H_2 in solution (10% in the gas phase, which is equivalent to 72 μM H_2 in solution) promoted higher rates of C_2H_2 reduction than a saturating concentration of succinate (10 mM). Addition of both H_2 and succinate to the bacteroid suspensions resulted in

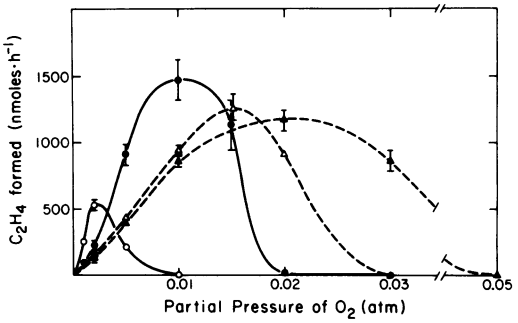

FIG. 1. *Effect of O_2, H_2, and succinate on C_2H_2 reduction by R. japonicum 122 DES bacteroids. C_2H_2 reduction assays were conducted as described in the text. The gas phase consisted of 0.1 atm of C_2H_2, 0.1 atm of H_2 when present, O_2 as indicated, and sufficient argon to obtain 1.0 atm. Each assay was started by adding the bacteroid suspension (7.94 mg, dry weight). Values reported are means of duplicates with the standard deviations indicated, and results are typical of those obtained in three experiments. C_2H_4 formation in the absence of succinate (○——○, ●——●); C_2H_4 formation in the presence of 10 mM succinate (△- - -△, ▲- - -▲); open symbols represent assays without H_2, and closed symbols represent assays with H_2.*

lower C_2H_2 reduction rates than were obtained with either H_2 or succinate alone (Fig. 1). The differences in C_2H_2 reduction rates between assays containing H_2 and those containing H_2 and succinate were dependent on the concentration of succinate added to the assay mixture (Table 1). At succinate concentrations of 1 mM or greater (Table 1), the C_2H_2 reduction activities of assays containing H_2 and succinate were lower than activities of assays containing H_2 alone. These differences, however, were not statistically significant. Adding both succinate and H_2 as substrates to bacteroid suspensions, however, extended the range of O_2 partial pressures at which C_2H_2 reduction occurred. The effect of H_2 on C_2H_2 reduction was specific for the H_2 uptake-positive *R. japonicum* strains as shown by experiments in which H_2 failed to enhance C_2H_2 reduction when H_2 uptake-negative USDA 117 bacteroids were used (Table 2).

The effect of succinate, with and without H_2, on C_2H_2 reduction rates and on the optimal partial pressure of O_2 for maximal C_2H_2 reduction was examined to determine the interaction between H_2-stimulated and succinate-stimulated C_2H_2 reduction (Table 1). Succinate at concentrations up to 1 mM increased C_2H_2 reduction rates by bacteroids, but concentrations greater than 1 mM caused a decline in activity (Table 1). This decrease may not be attributed entirely to excessive salts because equivalent concentrations of KCl had no appreciable effect. Increasing the succinate concentrations with and without H_2 increased the partial pressure of O_2 necessary for maximal C_2H_2 reduction rates (Table 1). In comparison with the rate of endogenous respiration of 0.35 μmol/h per mg (dry weight) of bacteroids, the addition of H_2, at 27 μM, increased the O_2 uptake rate about threefold to 1.03 μmol/h per mg (dry weight). The addition of 50 mM succinate increased the rate of O_2 uptake to 1.16 μmol/h per mg (dry weight). In the presence of 27 μM H_2 and 50 mM succinate, the rate of O_2 uptake increased to 1.65 μmol/h per mg (dry weight).

TABLE 1. *Effect of succinate in the presence and absence of H_2 on the maximal rates of C_2H_2 reduction and on the optimal concentration of O_2 for maximal C_2H_2 reduction*[a]

Succinate concentration (mM)	Maximal C_2H_2 reduction (nmol/h)		Optimal partial pressure O_2 for maximal C_2H_2 reduction (atm)	
	$-H_2$	$+H_2$	$-H_2$	$+H_2$
0	685 ± 34	1,560 ± 113	0.005	0.016
0.2	947 ± 147	1,605 ± 42	0.009	0.020
1.0	1,585 ± 187	1,515 ± 64	0.018	0.024
10.0	1,403 ± 28	1,375 ± 16	0.019	0.025
50.0	1,260 ± 24	1,245 ± 30	0.020	0.025

[a] A series of experiments analogous to that shown in Fig. 1 was performed at different potassium succinate concentrations. The maximal rates of C_2H_2 reduction and the optimal O_2 concentrations for maximal C_2H_2 reduction were determined for each succinate concentration. The C_2H_2 reduction assays were conducted as described in the text. The partial pressure of H_2 in the gas phase, when added, was 0.1 atm. A quantity of bacteroids equivalent to 5.7 mg (dry weight) was injected into each bottle to start each assay. The values for rates of C_2H_2 reduction are means of duplicate assays with standard deviations. Results are typical of those in three experiments.

TABLE 2. *Effect of H_2 and O_2 on C_2H_2 reduction by bacteroids from R. japonicum strain USDA 117*[a]

Composition of gas phase (atm)		C_2H_4 formed (nmol/h)
O_2	H_2	
0.002	0.0	39.8 ± 9.0
0.002	0.1	33.0 ± 9.0
0.005	0.0	5.8 ± 0.2
0.005	0.1	5.8 ± 0.2
0.01	0.0	1.8 ± 0.2
0.01	0.1	2.4 ± 0.8

[a] C_2H_2 reduction assays were conducted as described in the text. The gas phase consisted of 0.10 atm of C_2H_2, 0.10 atm of H_2, when present, O_2 as indicated, and argon to 1.0 atm. Bacteroids from strain USDA 117 (6.46 mg [dry weight]) were injected into the bottles to start the assay. Bacteroids were prepared anaerobically. Values reported are means of duplicate assays with standard deviations. Results are typical of those obtained in two additional experiments.

The effect of the partial pressure of C_2H_2 on the rate of C_2H_2 reduction by 122 DES bacteroids in the presence and absence of H_2 is shown in Fig. 2. The O_2-dependent and H_2-stimulated C_2H_2 reduction activities were both saturated at a C_2H_2 partial pressure of 0.1 atm. C_2H_2 did not inhibit the hydrogenase activity in this strain of bacteroids, which is in contrast with observations with *A. chroococcum* (27) and blue-green algae (6).

Effect of H_2 on ATP content. Direct measurement of the adenine nucleotide content of bacteroids of *R. japonicum* 122 DES shows that the addition of H_2 increased the steady-state level of ATP by 44% (Table 3). Typically, increases of 20 to 40% were found. The initial rates of increase in ATP content of bacteroids were similar with or without H_2 (Fig. 3). At all time periods during the experiment (Fig. 3), the ATP contents of bacteroids supplied with H_2 were greater than those of bacteroids incubated without H_2. In contrast, the addition of H_2 to the H_2 uptake-negative *R. japonicum* USDA 117 bacteroids had no consistent effect on the steady-state level of ATP (Table 3).

The inhibition of the H_2 uptake process by iodoacetate was less than the inhibition of endogenous respiration (Fig. 4). Iodoacetate, a general inhibitor of dehydrogenases, strikingly suppressed ATP formation that accompanied endogenous respiration, but ATP formation associated with H_2 oxidation was only partially in-

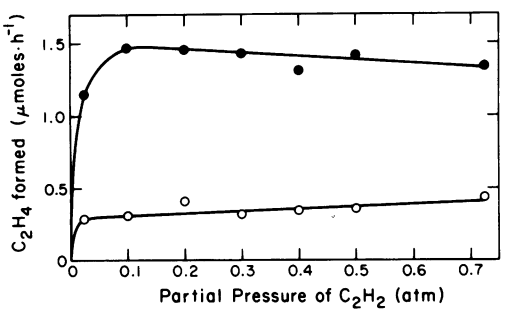

FIG. 2. *Effect of C_2H_2 and H_2 on the rates of C_2H_2 reduction by R. japonicum 122 DES bacteroids. C_2H_2 reduction assays were conducted as described in the text. The gas phase over the bacteroid suspensions consisted of varied partial pressures of C_2H_2 and 0.002 atm of O_2 in assays without H_2 and 0.01 atm of O_2 in assays with 0.1 atm of H_2. Sufficient argon was added to obtain 1 atm. Each assay contained bacteroid suspension equivalent to 7.9 mg (dry weight) of bacteroids. Each value presented is a mean of duplicate assays. Results are typical of those obtained in two experiments. C_2H_4 formation in the absence of H_2 (○) and in the presence of H_2 (●).*

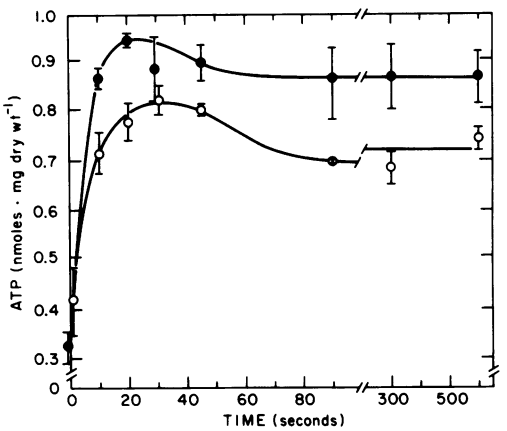

FIG. 3. *Effect of adding H_2 on the ATP content of R. japonicum strain USDA 122 DES bacteroids. The gas phase consisted of 0.20 atm of O_2, 0.10 atm of H_2 as indicated, and argon to 1.0 atm. Each assay contained the equivalent of 1.65 mg (dry weight) of aerobically prepared bacteroids. Assays were incubated for the indicated times. Values are means of duplicates with standard deviations indicated, and results are typical of those obtained in four experiments. ATP content in the absence of H_2 (○) and in the presence of H_2 (●).*

TABLE 3. *Effect of H_2 on the ATP content of R. japonicum bacteroids*[a]

Bacteroid strain	Conditions	Bacteroid ATP content (nmol/mg dry weight) with:		
		None	IA	CCCP
USDA 122 DES	$-H_2$	0.654 ± 0.025	0.134 ± 0.019	0.429 ± 0.062
	$+H_2$	0.939 ± 0.048	0.647 ± 0.042	0.406 ± 0.059
USDA 117	$-H_2$	0.625 ± 0.068	0.125 ± 0.021	0.298 ± 0.014
	$+H_2$	0.609 ± 0.038	0.130 ± 0.005	0.311 ± 0.015

[a] Assays for ATP content were determined in nominal 21-ml vials as described in the text. The concentrations of iodoacetate (IA) and CCCP were 50 mM and 25 μM, respectively. Assays with strain USDA 122 DES contained bacteroid suspensions equivalent to 1.66 mg (dry weight), and those with strain USDA 117 contained bacteroid suspensions of 2.23 mg (dry weight). Bacteroids were prepared aerobically. The gas phase consisted of 0.1 atm of H_2 (as indicated), 0.2 atm of O_2, and 0.7 atm of argon. Reactions were terminated after incubation for 5 min. Values are means of duplicate assays with standard deviations. The results are typical of those in four experiments for strain 122 DES and two experiments for strain 117.

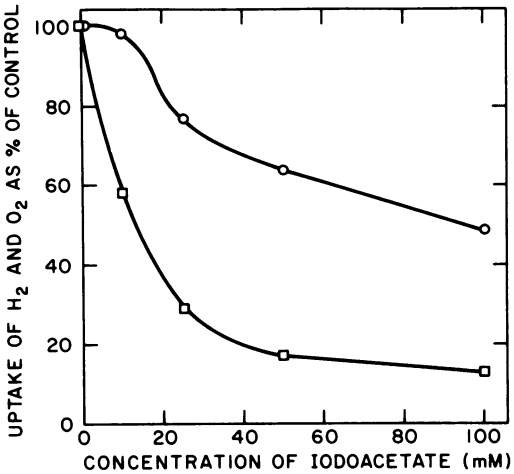

FIG. 4. *Effect of iodoacetate on H_2 uptake and on O_2 uptake in the absence of H_2. Amperometric assays were conducted as described in the text. Iodoacetate was incorporated into the buffer at the concentrations indicated. Aerobically prepared bacteroids were injected to start the assay (1.65 mg, dry weight). Values plotted are the relative activities compared to the uninhibited control. The values reported are means of duplicate assays and the results are typical of those obtained in three experiments. H_2 uptake* (○); *O_2 uptake in the absence of H_2* (□).

hibited by iodoacetate (Table 3). CCCP, an uncoupler of oxidative phosphorylation, at concentrations below 30 μM stimulated both endogenous and H_2-supported respiration (Fig. 5). Concentrations of CCCP greater than 50 μM inhibited both O_2-consuming reactions. Uptake of H_2 was inhibited by all concentrations of CCCP that were tested. Also, CCCP inhibited ATP formation associated with both endogenous respiration and H_2 oxidation (Table 3). When CCCP was added to suspensions, the ATP content of 122 DES bacteroids was not consistently affected by the addition of H_2. When both CCCP and iodoacetate were added, the level of ATP was reduced from 0.654 to 0.173 nmol/mg of dry bacteroids in the absence of H_2 and from 0.939 to 0.181 nmol/mg of dry bacteroids in the presence of H_2. The effects of iodoacetate and CCCP indicate that ATP formation in bacteroids may occur via oxidative phosphorylation.

Effect of H_2 on C_2H_2 reduction and ATP content. Experiments were conducted in which ATP contents and rates of C_2H_2 reduction of bacteroids in suspension were determined at a series of different O_2 partial pressures (Fig. 6a). In the absence of H_2, the ATP content roughly paralleled the increasing C_2H_2 reduction rates until the optimal partial pressure of O_2 for C_2H_2 reduction was achieved. Partial pressures of O_2 that were higher than the optimum decreased the rate of C_2H_2 reduction, but maintained the ATP content of bacteroids at a near constant level. Similar results were observed when H_2 was supplied. The ATP contents of bacteroids supplied with H_2 were lower than those without H_2 at partial pressures of O_2 that supported less than maximal C_2H_2 reduction activity. The reason for the lowered ATP contents and rates of C_2H_2 reduction in the presence of H_2 at suboptimal partial pressures of O_2 compared with assays without H_2 (Fig. 6a) is presently under investigation. Bacteroids provided with H_2 and partial pressures of O_2 that inhibited nitrogenase activity had higher ATP contents than comparable bacteroids without H_2. The ATP pool, however, increased, as might be expected when the partial pressure of O_2 reached a level that inhibited nitrogenase activity.

Addition of 50 mM succinate to reactions lacking H_2 (Fig. 6b) stimulated C_2H_2 reduction, increased the partial pressures of O_2 necessary for maximal C_2H_2 reduction, and suppressed the ATP content in suspensions maintained at suboptimal partial pressures of O_2 (Fig. 6b). When succinate was added, H_2 had no marked effect on the ATP content of the cells. The maximum C_2H_2 reduction rate measured in the presence of 50 mM succinate and H_2 (Fig. 6b) appears to be about 35% less than the rate observed in the

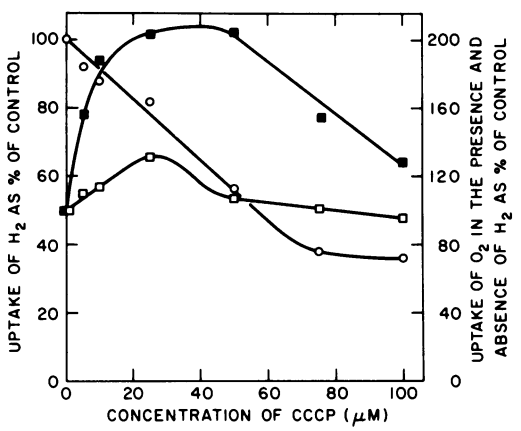

FIG. 5. *Effect of CCCP on H_2 uptake and O_2 uptake in the presence and absence of H_2. Amperometric assays were conducted as described in the text. CCCP was incorporated into the buffer at the concentrations indicated. The amount of aerobically prepared bacteroids added to each assay was equivalent to 1.65 mg (dry weight). Values plotted are the relative activities compared with the uninhibited control. Means of duplicate assays are reported, and results are typical of those obtained in three experiments. H_2 uptake,* (○); *O_2 uptake in the absence of H_2* (□) *and in the presence of H_2* (■).

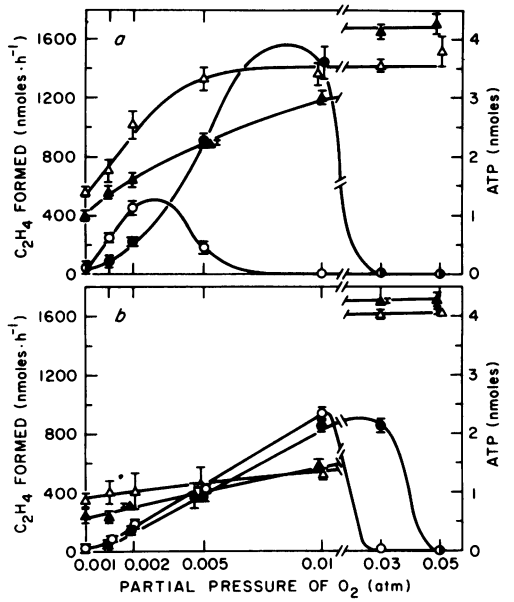

FIG. 6. *(a) Effect of O_2 on ATP content and C_2H_2 reduction by R. japonicum strain USDA 122 DES bacteroids. The gas phase consisted of 0.10 atm of C_2H_2, O_2 as indicated, 0.10 atm of H_2 when present, and argon to 1.0 atm. Bacteroids were injected (6.27 mg, dry weight) to start the assays. C_2H_4 formation was measured after 15 and 30 min of incubation. After 30 min of incubation, the reactions were terminated and prepared for nucleotide analysis. Values are means of duplicates with standard deviations indicated. Results are typical of three experiments. C_2H_4 formation (○, ●) and ATP content (△, ▲); open symbols represent assays without H_2, and closed symbols represent assays with H_2. (b) Effect of O_2 and H_2 plus O_2 on ATP content and C_2H_2 reduction by R. japonicum USDA 122 DES bacteroids in the presence of succinate. The experiment was similar to that described in (a) except that 50 mM succinate was incorporated into the buffer. The symbols used are the same as those listed in (a).*

presence of H_2 alone (Fig. 6a). The optimal partial pressure of O_2 for maximal C_2H_2 reduction in assays containing 50 mM succinate was 0.020 atm in the absence of H_2 and 0.025 atm in the presence of H_2 (Table 2). These partial pressures of O_2 were not included in Fig. 6, but the C_2H_2 reduction rates observed at these partial pressures of O_2 are consistent with those recorded in Table 2.

H_2-dependent C_2H_2 reduction. H_2 stimulated C_2H_2 reduction above the rates obtained when endogenous substrates were utilized (Fig. 6). Iodoacetate prevented an increase in ATP content of bacteroids supported by endogenous respiration (Table 3) and as expected inhibited C_2H_2 reduction (Table 4). The oxidation of H_2 increased the ATP content of bacteroids (Table 3) and consequently restored C_2H_2 reduction activity (Table 4) in iodoacetate-treated bacteroids. At 25 μM iodoacetate and 0.01 atm of O_2, H_2 restored about 40% of the activity relative to the uninhibited control. Higher concentrations of iodoacetate caused further suppression of H_2-supported C_2H_2 reduction. Thus, ATP generated by H_2 oxidation supported N_2 fixation.

TABLE 4. *Effect of H_2 and iodoacetate on C_2H_2 reduction of R. japonicum 122 DES bacteroids at different partial pressures of O_2*[a]

Iodoacetate concn (mM)	Conditions	C_2H_2 formed (nmol/h) at O_2 partial pressures (atm): 0.002	0.005	0.010
0	$-H_2$	488.2 ± 6.6	687.8 ± 68.6	81.6 ± 2.8
0	$+H_2$	462.6 ± 3.4	902.2 ± 14.2	1,590.0 ± 87.8
25	$-H_2$	12.8 ± 2.4	7.6 ± 0.8	1.8 ± 0.2
25	$+H_2$	57.6 ± 8.8	269.4 ± 15.0	661.2 ± 1.6
75	$-H_2$	4.4 ± 0.4	2.6 ± 0.2	1.2 ± 0.4
75	$+H_2$	7.8 ± 1.4	48.2 ± 1.4	186.0 ± 12.0

[a] C_2H_2 reduction assays were performed as described in the text except that iodoacetate was incorporated into the buffer where indicated. Gas phase contained 0.1 atm of C_2H_2, O_2 as indicated, 0.1 atm of H_2, where added, and argon to 1.0 atm. Bacteroids were prepared anaerobically. Bacteroid suspension equivalent to 6.09 mg (dry weight) was added to each assay. Values are means of duplicates with standard deviations and are typical of two experiments.

DISCUSSION

It is well established that most nodulated legumes evolve H_2 during the N_2 fixation process (23). Also, there is convincing evidence that those bacteroids that do not evolve H_2 from nodules possess a hydrogenase system that participates in an H_2-recycling process. *R. japonicum* 122 DES is a good example of a strain that possesses a very active hydrogenase system in bacteroids and recycles all the H_2 produced from the N_2 fixation reaction. In Table 1, the maximal rate of C_2H_2 reduction is 282 nmol of C_2H_2 reduced per h per mg (dry weight). If 25% of the electron flow through nitrogenase is used for H_2 evolution under N_2 reducing conditions (23), then the rate of H_2 evolution expected from the nitrogenase of these bacteroids is 71 nmol of H_2 evolved per h per mg (dry weight). The rate of H_2 oxidation by 122 DES bacteroids is typically 2 μmol/h per mg (dry weight). Thus, the hydrogenase system has the capacity to consume H_2 at more than 25 times the rate that H_2 is normally evolved from the nitrogenase system. From these data it is obvious why nodules formed by *R. japonicum* 122 DES evolve little or no H_2.

The addition of H_2 or succinate to *R. japonicum* bacteroid suspensions that contain the hy-

drogenase system strikingly stimulated the rate of O_2 uptake (Table 1). This response is consistent with Dalton and Postgate's (10) discussion of respiratory protection of the nitrogenase system. Associated with increased respiratory rates in the presence of H_2 was an increase in nitrogenase activity as measured by C_2H_2 reduction. The addition of either H_2 or succinate not only increased nitrogenase activity, but allowed the nitrogenase system to function at higher O_2 partial pressures than were possible when only endogenous substrates were utilized (Fig. 1). The H_2-recycling system in these bacteroids, therefore, acts as a buffer against changes in partial pressures of O_2 that may occur during senescence or conditions of plant stress. Also, it seems reasonable to postulate benefits from the H_2-recycling process when nodules are young and contain insufficient leghemoglobin to protect the nitrogenase complex from excessive O_2.

The addition of H_2 to bacteroids increased their steady-state level of ATP by 20 to 40%. When endogenous respiration was suppressed by iodoacetate, H_2 increased the steady-state level of ATP by 500%. H_2-dependent ATP formation occurred in *R. japonicum* bacteroids via oxidative phosphorylation as shown by its absolute O_2 requirement (23) and its inhibition by CCCP (Table 3 and reference 1).

For the H_2-oxidizing system of *R. japonicum* bacteroids to increase the efficiency of the N_2 fixation process (in addition to respiratory protection), the ATP formed by H_2 oxidation must be utilized to support the nitrogenase system or related reactions. In Fig. 6, increasing the O_2 partial pressures provided an increasing steady-state level of ATP and increased nitrogenase activity. Appleby et al. (1) have previously shown that increasing O_2 partial pressures increased the steady-state level of ATP and increased C_2H_2 reduction. In agreement with the reports of Appleby et al. (1), we also have observed a direct correlation between ATP/ADP ratio and nitrogenase activity in *R. japonicum* bacteroids (data not presented). When ATP formation from endogenous substrate utilization was suppressed by iodoacetate (Table 4), the addition of H_2 strikingly stimulated nitrogenase activity. This is convincing evidence that the ATP needed for nitrogenase activity can be provided by H_2 oxidation (Table 4).

It has been reported (5) that the H_2-oxidizing system in blue-green algae may provide both ATP and reductant for the nitrogenase system. Also, there is evidence that H_2 utilization in *A. chroococcum* may produce the reductant for nitrogenase (27). In the experiment reported here (Table 4) with *R. japonicum* bacteroids, the possibility exists that reductant for nitrogenase was supplied by reactions that were insensitive to inhibition by iodoacetate. Whether H_2 may serve as a reductant for the nitrogenase in *R. japonicum* bacteroids remains unanswered.

ACKNOWLEDGMENTS

We express gratitude to M. G. Yates and J. R. Postgate for stimulating and enlightening discussions and to Sterling Russell, Joe Hanus, and Wha-gi Lee for expert technical assistance. D.W.E. expresses his thanks to the Rockefeller Foundation for postdoctoral support. T.R.A. wishes to thank the Program of Cultural Cooperation between the United States and Spain for support. This research was supported by National Science Foundation grant 77-08784 and the Oregon Agricultural Experiment Station.

LITERATURE CITED

1. **Appleby, C. A., G. L. Turner, and P. K. Macnicol.** 1975. Involvement of oxyleghaemoglobin and cytochrome P-450 in an efficient oxidative phosphorylation pathway which supports N_2 fixation in rhizobium. Biochim. Biophys. Acta **387**:461-474.
2. **Bergersen, F. J., and G. L. Turner.** 1967. Nitrogen fixation by the bacteroid fraction of breis of soybean root nodules. Biochim. Biophys. Acta **141**:507-515.
3. **Bergersen, F. J., G. L. Turner, and C. A. Appleby.** 1973. Studies of the physiological role of leghemoglobin in soybean root nodules. Biochim. Biophys. Acta **292**: 271-282.
4. **Bethlenfalvay, G. J., and D. A. Phillips.** 1977. Effect of light intensity on efficiency of carbon dioxide and nitrogen reduction in *Pisum sativum* L. Plant Physiol. **60**: 868-871.
5. **Bothe, H., J. Tennigkeit, and G. Eisbrenner.** 1977. The utilization of molecular hydrogen by the blue-green alga *Anabaena cylindrica*. Arch. Microbiol. **114**:43-49.
6. **Bothe, H., J. Tennigkeit, G. Eisbrenner, and M. G. Yates.** 1977. The hydrogenase-nitrogenase relationship in the blue-green alga *Anabaena cylindrica*. Planta **133**:237-242.
7. **Carter, K. R., N. T. Jennings, F. J. Hanus, and H. J. Evans.** 1978. Hydrogen evolution and uptake by nodules of soybeans inoculated with different strains of *Rhizobium japonicum*. Can. J. Microbiol. **24**:307-311.
8. **Ching, T. M., and K. K. Ching.** 1972. Content of adenosine phosphates and adenylate energy charge in germinating ponderosa pine seeds. Plant Physiol. **50**: 536-540.
9. **Ching, T. M., S. Hedtke, and W. Newcomb.** 1977. Isolation of bacteria, transforming bacteria and bacteroids from soybean nodules. Plant Physiol. **60**:771-774.
10. **Dalton, H., and J. R. Postgate.** 1969. Effect of oxygen on growth of *Azotobacter chroococcum* in batch and continuous cultures. J. Gen. Microbiol. **54**:463-473.
11. **Dixon, R. O. D.** 1967. Hydrogen uptake and exchange by pea root nodules. Ann. Bot. **31**:179-188.
12. **Dixon, R. O. D.** 1968. Hydrogenase in pea root nodule bacteroids. Arch. Mikrobiol. **62**:272-283.
13. **Dixon, R. O. D.** 1972. Hydrogenase in legume root nodule bacteroids: occurrence and properties. Arch. Mikrobiol. **85**:193-201.
14. **Hyndman, L. A., R. H. Burris, and P. W. Wilson.** 1953. Properties of hydrogenase from Azotobacter vinelandii. J. Bacteriol. **65**:522-531.
15. **Klucas, R. V., B. Koch, S. A. Russell, and H. J. Evans.** 1968. Purification and some properties of the nitrogenase from soybean (*Glycine max*. Merr.) nodules. Plant Physiol. **43**:1906-1912.
16. **Kuykendall, L. D., and G. H. Elkan.** 1976. *Rhizobium japonicum* derivatives differing in nitrogen-fixing efficiency and carbohydrate utilization. Appl. Environ. Microbiol. **32**:511-519.

17. **McCrae, R. E., J. Hanus, and H. J. Evans.** 1978. Properties of the hydrogenase system in *Rhizobium japonicum* bacteroids. Biochem. Biophys. Res. Commun. **80**:384–390.
18. **O'Gara, F., and K. T. Shanmugam.** 1978. Mutant strains of clover rhizobium (*Rhizobium trifolii*) that form nodules on soybean (*Glycine max*). Proc. Natl. Acad. Sci. U.S.A. **75**:2343–2347.
19. **Peterson, R. B., and R. H. Burris.** 1978. Hydrogen metabolism in isolated heterocysts of *Anabaena* 7120. Arch. Microbiol. **116**:125–132.
20. **Phelps, A. S., and P. W. Wilson.** 1941. Occurrence of hydrogenase in nitrogen-fixing organisms. Proc. Soc. Exp. Biol. Med. (N.Y.) **47**:473–476.
21. **Rigaud, J., F. J. Bergersen, G. L. Turner, and R. M. Daniel.** 1973. Nitrate dependent anaerobic acetylene reduction and nitrogen fixation by soybean bacteroids. J. Gen. Microbiol. **77**:137–144.
22. **Ruiz-Argüeso, T., J. Hanus, and H. J. Evans.** 1978. Hydrogen production and uptake by pea nodules as affected by strains of *Rhizobium leguminosarum*. Arch. Microbiol. **116**:113–118.
23. **Schubert, K. R., and H. J. Evans.** 1976. Hydrogen evolution: a major factor affecting the efficiency of nitrogen fixation in nodulated symbionts. Proc. Natl. Acad. Sci. U.S.A. **73**:1207–1211.
24. **Smith, L. A., S. Hill, and M. G. Yates.** 1976. Inhibition by acetylene of conventional hydrogenase in nitrogen-fixing bacteria. Nature (London) **262**:209–210.
25. **Tel-Or, E., L. W. Luijk, and L. Packer.** 1977. An inducible hydrogenase in cyanobacteria enhances N_2 fixation. FEBS Lett. **78**:49–52.
26. **Vincent, J. M.** 1970. A manual for the practical study of the root-nodule bacteria, p. 116. International biological programme, vol. 15. Blackwell Scientific Publications, Oxford.
27. **Walker, C. C., and M. G. Yates.** 1978. The hydrogen cycle in nitrogen-fixing *Azotobacter chroococcum*. Biochimie **60**:225–231.
28. **Wittenberg, J. B., F. J. Bergersen, C. A. Appleby, and G. L. Turner.** 1974. Facilitated oxygen diffusion. J. Biol. Chem. **249**:4057–4066.